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The small protein CydX is required for function of cytochrome *bd* oxidase in *Brucella abortus*

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A large number of hypothetical genes potentially encoding small proteins of unknown function are annotated in the *Brucella abortus* genome. Individual deletion of 30 of these genes identified four mutants, in *BAB1_0355*, *BAB2_0726*, *BAB2_0470*, and *BAB2_0450* that were highly attenuated for infection. *BAB2_0726*, an YbgT-family protein located at the 3′ end of the *cydAB* genes encoding cytochrome *bd* ubiquinal oxidase, was designated *cydX*. A *B. abortus cydX* mutant lacked cytochrome *bd* oxidase activity, as shown by increased sensitivity to H_2O_2, decreased acid tolerance and increased resistance to killing by respiratory inhibitors. The C terminus, but not the N terminus, of CydX was located in the periplasm, suggesting that CydX is an integral cytoplasmic membrane protein. Phenotypic analysis of the *cydX* mutant, therefore, suggested that CydX is required for full function of cytochrome *bd* oxidase, possibly via regulation of its assembly or activity.

Keywords: cytochrome oxidase, terminal electron acceptor, mutant screen, peptide

INTRODUCTION

Genes encoding small proteins are not well-characterized in the genomes of bacteria. An examination of the sequenced genomes shows that these small genes are not consistently annotated across related genomes, which raises the question of whether they encode functional genes. Further, the small size of the coding genes reduces the probability of identifying their functions in transposon mutagenesis screens. In bacteria, relatively few small proteins have been characterized. These include the toxin peptides of toxin/antitoxin systems, which insert into membranes and cause damage (Gerdes et al., 1986), and peptides that functions as "connectors" of two-component signal transduction systems (Eguchi et al., 2011). Recent studies in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium have identified several small proteins whose expression had previously been overlooked in proteomic and genomic studies (Alix and Blanc-Potard, 2009; Hemm et al., 2010). One of these proteins, MgtR, modulates degradation of MgtC, a cytoplasmic protein involved in replication of *S. typhimurium* within host cells, by the FtsH protease, suggesting a role for MgtR in adaptation to the host environment (Alix and Blanc-Potard, 2008). A second peptide found to target the activity of FtsH protease, SpoVM, has been identified in *Bacillus subtilis* (Cutting et al., 1997). In *Agrobacterium tumefaciens*, a close phylogenetic relative of the *Brucella* species, a small protein VirE1 has been shown to serve as a secretion chaperone for the Type IV-exported single-strand binding protein VirE2 (Deng et al., 1999). Given their potential role in virulence-associated functions, we determined the role in intracellular survival of 30 genes with the potential to encode proteins of <15 kDa in the intracellular pathogen *Brucella abortus*. One of the proteins identified in this screen, BAB2_0726, was encoded in the 3′ region of *cydAB*, encoding cytochrome *bd* oxidase. This terminal oxidase of the respiratory chain is expressed by *B. suis* inside macrophages, the preferred intracellular niche *in vivo* (Loisel-Meyer et al., 2005), and contributes to intracellular replication of *B. abortus*, *B. suis*, and *B. melitensis* (Endley et al., 2001; Loisel-Meyer et al., 2005; Wu et al., 2006). We, therefore, focused our efforts on the contribution of BAB2_0726 to the function of cytochrome *bd* oxidase.

MATERIALS AND METHODS

BACTERIAL STRAINS, MEDIA, AND CULTURE CONDITIONS

Escherichia coli TOP10 cells were used for all cloning steps and transformation of PCR products cloned into the pCR2.1 TOPO vector (Invitrogen). All *E. coli* strains were grown in Luria-Bertani (LB) broth. Antibiotics carbenicillin (Carb) 100 μg/ml, kanamycin (Kan), 100 μg/ml or chloramphenicol (Cm), 30 μg/ml were used as appropriate. *Brucella abortus* biovar 1 strain 2308, a virulent strain originally isolated from cattle, was used for generation of mutants. Bacterial inocula used for infection of mice were cultured on tryptic soy agar (TSA) plus 5% defibrinated sheep blood. The antibiotics Carb 100 μg/ml, Kan, 50 μg/ml; Cm, 15 μg/ml were added as needed. All work with live *B. abortus* was performed at biosafety level 3. Bacterial strains and plasmids used in this study are listed in **Table 1**.

ALLELIC EXCHANGE MUTAGENESIS

A previously reported three-step cloning strategy (Sun et al., 2005) was used to construct mutants for small open reading frames (**Figure A1**). Briefly, an upstream fragment (with a *Sma*I site in reverse primer) and a downstream fragment (with a *Sma*I site in forward primer and a *Pst*I site in reverse primer), both

Table 1 | Strains and plasmids used in this work.

Strain or plasmid	Description	Reference or source
PLASMIDS		
pCR2.1	PCR TOPO cloning vector	Invitrogen
pUC4Kixx	1.3 Kb Kan cassette as *Sma*I fragment, Amp^R, Kan^R	Pharmarcia
pBBR1MCS4	Broad-host range vector confers Amp^R	Kovach et al., 1995
pFlagTEM1	Cloning vector to express flagged TEM1 (FT) fusion under control of trc promoter, Cm^R	Raffatellu et al., 2005
pCydX/FT	Expresses CydX::FT from trc promoter, Cm^R	This study
pFT/CydX	Expresses FT::CydX from trc promoter, Cm^R	This study
pGST/FT	Expresses GST::FT from trc promoter, Cm^R	Raffatellu et al., 2005
pFT/GST	Expresses FT::GST fromtrc promoter, Cm^R	de Jong et al., 2008
pGroEL/dsRed	Expresses DsRed from *Brucella* GroEL promoter, Cm^R	This study
pGroEL/CydX	Expresses CydX from *Brucella* GroEL promoter, Cm^R	This study
pGroEL/CydB	Expresses CydB from *Brucella* GroEL promoter, Cm^R	This study
pGroEL/CydBX	Expresses CydBX from *Brucella* GroEL promoter, Cm^R	This study
pBBR1Flag	Cloning vector to express flagged fusion under *Brucella* constitutive promoter, Cm^R	This study
pCydX/Flag	Expresses CydX::Flag from *Brucella* constitutive promoter, Cm^R	This study
BRUCELLA		
B. abortus 2308	Wild type	
ADH3	Δ*vir*B2 (non polar) in *B. abortus* 2308	den Hartigh et al., 2004
BA41	Δ*vir*B1::Tn5 in *B. abortus* 2308	Hong et al., 2000
BA582	Δ*cyd*B::Tn5 in *B. abortus* 2308	Endley et al., 2001
	ΔBAB1_0061::Kan in *B. abortus* 2308	This study
	ΔBAB1_0355::Kan in *B. abortus* 2308	This study
	ΔBAB1_0513::Kan in *B. abortus* 2308	This study
	ΔBAB1_0581::Kan in *B. abortus* 2308	This study
	ΔBAB1_0626::Kan in *B. abortus* 2308	This study
	ΔBAB1_0743::Kan in *B. abortus* 2308	This study
	ΔBAB1_0992::Kan in *B. abortus* 2308	This study
	ΔBAB1_1106::Kan in *B. abortus* 2308	This study
	ΔBAB1_1194::Kan in *B. abortus* 2308	This study
	ΔBAB1_1204::Kan in *B. abortus* 2308	This study
	ΔBAB1_1262::Kan in *B. abortus* 2308	This study
	ΔBAB1_1390::Kan in *B. abortus* 2308	This study
	ΔBAB1_1392::Kan in *B. abortus* 2308	This study
	ΔBAB1_1743::Kan in *B. abortus* 2308	This study
	ΔBAB1_1748::Kan in *B. abortus* 2308	This study
	ΔBAB1_1939::Kan in *B. abortus* 2308	This study
	ΔBAB1_2041::Kan in *B. abortus* 2308	This study
	ΔBAB2_0160::Kan in *B. abortus* 2308	This study
	ΔBAB2_0170::Kan in *B. abortus* 2308	This study
	ΔBAB2_0862::Kan in *B. abortus* 2308	This study
	ΔBAB2_0761::Kan in *B. abortus* 2308	This study
	ΔBAB2_0726::Kan in *B. abortus* 2308 (Δ*cyd*X::Kan)	This study
	ΔBAB2_0470::Kan in *B. abortus* 2308	This study
	ΔBAB2_0450::Kan in *B. abortus* 2308	This study
	ΔBAB2_0415::Kan in *B. abortus* 2308	This study
	ΔBAB2_0400::Kan in *B. abortus* 2308	This study
	ΔBAB2_0232::Kan in *B. abortus* 2308	This study
	ΔBAB2_1072::Kan in *B. abortus* 2308	This study
	ΔBAB2_1087::Kan in *B. abortus* 2308	This study
	ΔBAB2_1036::Kan in *B. abortus* 2308	This study
	B. abortus 2308 with pCydX/Flag	This study
	B. abortus 2308 with pBBR1MCS4	This study
	B. abortus 2308 with pFlagTEM1	de Jong et al., 2008

(Continued)

Table 1 | Continued

Strain or plasmid	Description	Reference or source
	B. abortus 2308 with pCydX/FT	This study
	B. abortus 2308 with pFT/CydX	This study
	B. abortus 2308 with pGST/FT	This study
	B. abortus 2308 with pFT/GST	de Jong et al., 2008
	Δ*cyd*X::Kan with pGroEL/dsRed	This study
	Δ*cyd*X::Kan with pGroEL/CydX	This study
	Δ*cyd*X::Kan with pCydX/Flag	This study
	BA582 with pGroEL/CydX	This study
	BA582 with pGroEL/CydB	This study
	BA582 with pGroEL/CydBX	This study

measuring about 600–1200 bp, were amplified and cloned in pCR2.1. The orientation of the upstream fragment was determined by PCR using one primer specific for the insert and one primer specific for the vector (for example T7 promoter primer) to identify a clone with the *Sma*I site located at the f1 origin end of the multiple cloning site in TOPO cloning vector pCR2.1. The *Sma*I/*Pst*I digested downstream fragment was then cloned next to the upstream fragment by using ampicillin for selection marker since the *Sma*I/*Pst*I double digestion of vector cleaves a 1.2 Kb fragment from pCR2.1 backbone including part of kanamycin resistance gene. A 1.3 Kb *Sma*I fragment of pUC4KIXX (Pharmacia) containing the Tn*5* kanamycin resistance gene was then cloned into the *Sma*I site to generate a construct for the allelic exchange of candidate gene with a deleted copy replaced by a kanamycin resistance gene. These plasmids were introduced into *B. abortus* 2308 by electroporation. Recombinants were screened for kanamycin resistance and carbenicillin sensitivity. Deletion of target genes was confirmed by PCR or/and southern blot.

RNA PREPARATION AND RT-PCR

B. abortus strains were grown for 48 h in tryptic soy broth (TSB) in parafilm-sealed tubes with minimal headspace to limit oxygen availability. The cells were harvested by centrifugation at 5000 × *g*. Total RNA was isolated using the Aurum Mini Kit (Bio-rad). RNA samples were digested with RNase-free DNase I (Invitrogen) and quantified using a NanoDrop spectrophometer (Thermo Scientific) at 260/280 nm. Reverse transcription was carried out using a standard protocol and MMLV Reverse Transcriptase (Invitrogen). Primer pairs used to amplify *cydA* (247 bp), *cydB* (243 bp), and *cydX* (204 bp) are listed in **Table A1**. PCR primers (2 μM) and cDNAs were added in PCR SuperMix (Invitrogen), and heated to 95°C for 5 min. Reactions omitting the reverse transcriptase served as negative controls. The PCR cycle consisted of 94°C for 10 s (denaturation), 52–55°C for 30 s (annealing), and 72°C for 10 s (extension), and this was repeated 30 times. PCR products were visualized after agarose gel (1.4%) electrophoresis and SYBR staining (Invitrogen).

MACROPHAGE KILLING ASSAY

The mouse macrophage-like cell line J774A.1, obtained from ATCC, was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, and 1 mM Glutamine (DMEMsup). For macrophage killing assays, 24-well microtiter dishes were seeded with macrophages at a concentration of 1–2 ×105 cells/well in 0.5 ml of DMEMsup and incubated over night at 37°C in 5% CO_2. Inocula were prepared by growth with shaking in TSB for 24 h, then subsequent dilution in DMEMsup to a concentration of 4×10^7 colony-forming units (CFU)/ml. Approximately 2×10^7 bacteria in 0.5 ml of DMEMsup, containing single or a 1:1 mixture of wild type and mutant, were added to each well of macrophages.Microtiter dishes were centrifuged at 250 × *g* for 5 min at room temperature in order to synchronize infection. Cells were incubated for 20 min at 37°C in 5% CO_2, free bacteria were removed by three washes with phosphate-buffered saline (PBS). DMEMsup plus 50 μg gentamicin per ml was added to the wells, and the cells were incubated at 37°C in 5% CO_2. After 1 h, the DMEMsup plus 50 μg/ml gentamicin was replaced with medium containing 25 μg/ml gentamicin. Wells were sampled at 48 h after infection by aspirating the medium, lysing the macrophages with 0.5 ml of 0.5% Tween-20 and rinsing each well with 0.5 ml of PBS. Viable bacteria were quantified by dilution in sterile PBS and plating on TSA or/and TSA + Kan. Competitive Index, which were adjusted by inocula CFU, were calculated as Mutant CFU/WT CFU per well.

COMPETITIVE INFECTION IN MICE

Female BALB/c ByJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and used at an age of 6–10 weeks. For mixed infection experiments, groups of 2–5 mice were inoculated intraperitoneally with 0.5 ml of PBS containing 2×10^5 CFU of a 1:1 mixture of *B. abortus* wild type and the isogenic mutant. Infected mice were held in microisolator cages in a Biosafety Level 3 facility. At four weeks post-infection, mice were euthanized by CO_2 asphyxiation and the spleens were collected aseptically at necropsy. Spleens were homogenized in 3 ml of PBS and serial dilutions of the homogenate plated on TSA and TSA containing kanamycin, for enumeration of mutant and wild type CFU. Competitive Index was calculated as Mutant CFU/WT CFU recovered from the spleen divided by Mutant CFU/WT CFU in the inoculum. Animal experiments were approved by the UC Davis Institutional Animal Care and Use Committee.

ASSAY FOR LOSS OF STATIONARY PHASE VIABILITY

B. abortus cells, freshly re-suspended from TSA plates, were washed once with PBS and diluted into 5 ml TSB or modified minimal E-medium (Kulakov et al., 1997) pH 7.0 with starting $OD_{600} = 0.01$. Bacteria were grown in a shaking incubator at optimal condition of 200 rpm, 37°C, and 5% CO_2. At time points up to seven days bacterial CFU were enumerated by serial dilution and plating on TSA. Loss of viability during stationary phase was defined as a reduction in bacterial numbers after 72 h incubation. Experiments were repeated at least three times and produced similar results. Results of a representative experiment are shown.

ACID TOLERANCE TEST

After 24 h of growth in E-medium pH 7.0, bacteria were washed once with PBS and re-suspended in the same volume of E-medium adjusted to pH 7.0, or to pH 5.0 or 3.5 with 1 M Citric Acid. After 5 h incubation, CFU were determined by serial diluting and plating on TSA. Acid tolerance was measured by comparing growth at pH 3.5 or 5.0 to growth at pH 7.0. Experiments were repeated at least three times and produced similar results, and representative results are shown.

SENSITIVITY TO NICKEL SULFATE AND SODIUM AZIDE

To test *Brucella* sensitivity to heavy metal and respiratory chain inhibitor, a previously described method was used with modification (Endley et al., 2001). Log phase bacteria, obtained after 24 h growth in E-medium pH 7.0, were serially diluted and plated on TSA or TSA supplemented with 0.15 mM $NiSO_4$ and 0.15 mM NaN_3. CFU were counted after five days' incubation. Experiments were repeated at least three times, with similar results. Data from a representative experiment are shown.

MEASUREMENT OF HYDROGEN PEROXIDE SENSITIVITY

To measure hydrogen peroxide sensitivity, a previously described method was used (Elzer et al., 1994). Briefly, bacteria lawns from exponential growth phase were made by plating 100 µl *Brucella* growing in E-medium pH 7.0 on TSA plates. A small sterile disk of Whatman paper containing 5 µl 30% H_2O_2 was placed in the middle of each lawn. After five days' incubation, the zone of bacterial clearance was measured. Experiments were repeated at least three times with triplicate plates. Results from a single representative experiment are shown.

COMPLEMENTATION OF THE *cydX* MUTANT

In order to restore a functional copy of *cydX* in the mutant, a previously described *groEL* promoter (Gor and Mayfield, 1992; Lin et al., 1992) corresponding to *Brucella abortus* 2308 chromosome II DNA coordinates 184149–184551 with engineered restriction enzyme sites was amplified using primers GroEL-P-F and GroEL-P-R and cloned into the pCR2.1 TOPO cloning vector. The insert was excised using *Sph*I and *Nde*I and ligated into broad host range cloning vector pFlagTEM1 (GeneBank accession number EU44730) (Raffatellu et al., 2005; Sun et al., 2007) to replace $lacI^q$ and the *trc* promoter. *cydX*, *cydB*, and *cydBX* were amplified from *Brucella abortus* 2308 genomic DNA, respectively, cloned into TOPO vector and then introduced just downstream of the *groEL* promoter using *Nde*I and *Pst*I, to generate plasmids pGroEL/CydX, pGroEL/CydB, and pGroEL/CydBX. To show functionality of the GroEL promoter in *B. abortus*, a DsRed gene, encoding a red fluorescent protein (Shaner et al., 2004), was also cloned in the same way to generate pGroEL/dsRed. In order to monitor CydX expression, a plasmid with a 3×FLAG tag fused at the C terminus of CydX was constructed so that CydX expression as a tagged protein could be conveniently detected by using anti-FLAG monoclonal antibody (Sigma). Briefly, to ensure 3×FLAG—tagged protein expression in *Brucella*, a promoter demonstrated previously to be constitutively active under *in vitro* growth conditions (Eskra et al., 2001), corresponding to *B. abortus* 2308 chromosome II DNA coordinates 1045014 and 1045238 was amplified with primers Promoter-F and Promoter-R. The 226 bp amplicon flanked by *Kpn*I-*Hpa*I and *Nde*I-*Sal*I was cloned into pCR2.1 TOPO vector and the orientation was chosen in such a way that double digestion with *Sal*I/*Sac*I did not excise the insert. 3×FLAG with two stop codons, amplified from pSUB11 (Uzzau et al., 2001) using primer pair of Flag-F and Flag-R, digested with *Sal*I and *Sac*I, was then ligated with constitutive promoter $P_{BMEII0192}$ in pCR2.1 TOPO vector. The 270 bp of "$P_{BMEII0192}$-*Nde*I-*Sal*I-3×Flag," digested as *Kpn*I/*Sac*I fragment, was inserted into the same sites of pBBR1MCS (Elzer et al., 1994) to give rise to pBBR1Flag. Plasmid pCydX/Flag was made by cloning *cydX*, which was amplified from the *B. abortus* genome, TOPO-cloned into pCR2.1, and excised as *Nde*I/*Xho*I fragment, into *Nde*I/*Sal*I sites of pFlag (*Xho*I and *Sal*I sites are compatible). All constructs were confirmed by DNA sequencing to ensure correct insertion and/or in frame expression of fusion proteins. All primers were used in this work are listed in **Table A1**.

DETERMINING CydX LOCALIZATION AND ORIENTATION USING β-LACTAMASE AS A REPORTER

pFlagTEM1 (Raffatellu et al., 2005; Sun et al., 2007) was used to express β-lactamase fusions to CydX. To express 3×FLAG-tagged β-lactamase as an fusion to the C-terminus of CydX (CydX::TEM1) *cydX* from *B. abortus* 2308 was amplified without STOP codon, inserted into pCR2.1 and excised as an *Nde*I and *Sal*I fragment. This fragment was then cloned into plasmid pFlagTEM1 using *Nde*I and *Xho*I. Similarly, to fuse 3×FLAG-tagged β-lactamase to the N terminus of CydX, *cydX* was amplified and cloned into pFlagTEM1 using *Xba*I and *Pst*I to express TEM1::CydX. Other fusions were made in the same way or have been already described (**Table 1**). All constructs were sequenced to ensure in frame expression as Flag tagged TEM-1 fusion proteins.

Fresh bacteria growing on plates were resuspended in TSB. Serial dilutions were made after OD_{600} was measured. 10 µl of bacteria containing about 10^3 CFU were spotted on TSA in 96-well plates with 100 µg/ml Carb, or 1 mM IPTG plus 15 µg/ml Cm, or IPTG plus Carb which concentrations range from 0 to 1 mg/ml. Plates were checked after five days' incubation to determine bacterial viability. Survival was scored as the maximum antibiotic concentration permitting growth of *B. abortus*.

SDS-PAGE AND WESTERN BLOTTING

To measure expression of 3×FLAG-tagged or/and β-lactamase fusion proteins, *B. abortus* was cultured in TSB at 37°C with shaking at 200 rpm. After 18 h the OD_{600} of the cultures ranged

from 1.2 to 1.5 and bacteria were pelleted and resuspended in 1X Laemmli sample buffer, heated at 100°C for 5 min, and the total protein equivalent to 1×10^8 CFU loaded per well was then electrophoresed on a 12–15% polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were blocked in 2% non-fat skim milk in PBS for an hour and then probed with Anti-FLAG Monoclonal antibody (1:5000, Sigma) or anti-β-lactamase monoclonal antibody (1:5000, QED Bioscience Inc.). Primary antibody binding was detected using a goat-anti-mouse antibody conjugated to horseradish peroxidase (HRP). HRP activity was detected with a chemiluminescent substrate (NEN). A previously described polyclonal antibody against the *B. abortus* protein Bcsp31 was used as a loading control (Sun et al., 2005).

RESULTS AND DISCUSSION

TARGETED MUTAGENESIS OF SMALL HYPOTHETICAL ORFs

Using the published genome sequences of *B. abortus* 2308, *B. suis* 1330, and *B. melitensis* 16 M, we identified ORFs with the potential to encode proteins smaller than 15 kDa. Thirty of these ORFS were selected for mutagenesis based on having no predicted function and lack of annotation in the genome of *A. tumefaciens* C58. Some of these proteins were assigned families of conserved proteins such as clusters of orthologous genes (COG) with only general functional predictions or families containing conserved domains of unknown function (DUF). Precise deletions of each ORF were constructed and marked with a kanamycin resistance gene (**Tables 1**, **2**). Mutations were confirmed by PCR, and/or by Southern blot using probes specific to the deleted gene. For 30 genes we could confirm inactivation (See **Figure A1** for details of the mutagenesis procedure).

MUTANTS BAB2_0726, BAB2_0470, AND BAB2_0450 ARE HIGHLY ATTENUATED BOTH IN CULTURED MACROPHAGES *IN VITRO* AND IN A MOUSE MODEL *IN VIVO.*

In order to determine whether any of the genes inactivated in the collection of mutants were required for intracellular survival we tested the ability of 30 mutants to survive and replicate for 48 h within cells of the macrophage-like line J774. All the mutants had similar growth rates as wild type *B. abortus* when growing in TSB (data not shown). Three mutants, BAB2_0726, BAB2_0470, and BAB2_0450 exhibited marked intracellular growth defects in a competitive infection against wild type in J774 macrophage-like cells (**Table 2**). Macrophage infection experiments were repeated with individual mutants (non-competitive infection) and exhibited the same attenuation phenotype as in mixed infection. After 48 h infection, the intracellular CFU of mutants BAB2_0726, BAB2_0470, and BAB2_0450 was decreased 2–3 orders of magnitude compared with wild type although they were less attenuated than ADH3 (*virB2*), a mutant defective in the type IV secretion system (den Hartigh et al., 2004) (**Table 2**).

We also investigated the role of these small hypothetical genes in persistent infection in a mouse model. To minimize the variation caused by different inoculum doses, we conducted competitive infection experiments, in which we inoculated mice IP with a 1:1 mixture of each mutant with wild type *B. abortus* 2308. Four weeks after inoculation, bacterial load in spleens were determined by differential plating on TSA with or without kanamycin and the competitive index was calculated as CFU of mutants/CFU of wild type and corrected for mutant/wild type ratio in each inoculum. Most of the mutants were recovered in a number similar to the wild type from the spleen of infected mice, suggesting that these mutants have equal ability to persist for up to four weeks. However, three mutants were attenuated compared with wild type, having competitive indices ranging from 0.018 to 0.001. Consistent with the attenuation phenotype observed in cultured mouse macrophages *in vitro,* mutants BAB2_0726, BAB2_0470, and BAB2_0450 were highly attenuated *in vivo* (**Table 2**). A fourth mutant, BAB1_0355, was found to be attenuated in the *in vivo* competitive infection assay, but not in J774 cells (**Table 2**). BAB1_0355 is related to LrgA of *Staphylococcus aureus*, which encodes a regulator of murein hydrolase (Groicher et al., 2000). BAB2_0450 is upstream of a predicted carbonic anhydrase, which has been characterized as a target for anti-*Brucella* drugs (Joseph et al., 2010), so its function may be related to that of the carbonic anhydrase, or its deletion may have a polar effect on carbonic anyhdrase expression. BAB2_0470 belongs to a family of proteins designated "S23 ribosomal proteins" which share similarity to proteins of unknown function encoded by an intervening sequence in the 23S ribosomal genes of *Leptospira* and *Coxiella burnetii* (Ralph and McClelland, 1993; Afseth et al., 1995).

BAB2_0726 IS LOCATED IN THE 3′ END OF *cyd* OPERON AND THE PHENOTYPE OF A DELETION MUTANT IS SIMILAR TO THAT OF *cydB* MUTANTS

The *in vitro* and *in vivo* attenuation phenotypes of mutants BAB2_0726, BAB2_0470, and BAB2_0450 raised the question of how these small hypothetical proteins could contribute to *Brucella* virulence. While analysis of BAB2_0470 and BAB2_0450 did not reveal any clues to their function, by looking the gene organization of BAB2_0726 in *Brucella abortus* 2308 genome we found that BAB2_0726 is located at the 3′ end of the *cydAB* operon, a gene cluster encoding subunits of a high affinity terminal oxidase of the oxygen respiratory chain (Endley et al., 2001) (**Figure 1A**). This gene organization pattern is well conserved in all sequenced *Brucella* genomes. The localization of a small peptide downstream of *cydB* was also reported in *Escherichia coli*, where it has been designated *orfC* or *ybgT* (Muller and Webster, 1997; Hemm et al., 2010). This finding led us to designate BAB2_0726 as *cydX*, since *cydC* (*BAB2_0729*) and *cydD* (*BAB2_0730*) immediately upstream of *cydA* were already assigned in *E. coli* to genes encoding a bacterial glutathione transporter (Pittman et al., 2005).

Since based on *in silico* analysis, *cydA*, *cydB*, and *cydX* are predicted to be transcribed together from a promoter upstream of *cydA*, we determined whether insertional activation of *cydB* would be polar on *cydX* expression (**Figure 1B**). To this end, RT-PCR was performed to assay expression of *cydA*, *cydB*, and *cydX* in *B. abortus* 2308 (WT) or an isogenic Tn*5* insertion in *cydB* (Endley et al., 2001). While a faint product for *cydA* was observed in both WT and the *cydB* mutant, neither *cydB* nor *cydX* amplification products were found in the *cydB* mutant, suggesting a polar effect of the *cydB* mutation on *cydX* expression. These results suggested that *cydX* is transcribed together with the cytochrome *bd* oxidase subunits encoded by *cydA* and *cydB*.

Table 2 | Small ORFs (<15 kDa) with no assigned function, which are highly conserved in *Brucella* species.

B. abortus 2308 ORF[a]	*B. suis* 1330 ORF[a]	*B. melitensis* 16M ORF[a]	Annotation[b]	*In vitro* CI[c]	*In vivo* CI[d]
BAB2_0068[e]	BRA0069	BMEII0025	Type IV secretion system protein virB1	0.0023	0.0001
BAB1_0061	BR0064	BMEI1881	hypothetical protein	0.7493	1.2039
BAB1_0355	BR0325	BMEI1597	COG1380 LrgA family protein [general function prediction only]	1.3655	0.0153
BAB1_0513	BR0488	BMEI1447	hypothetical protein	5.6537	1.0448
BAB1_0581	BR0557	BMEI1376	COG3654; prophage maintenance system killer protein [general function prediction only]	1.8387	0.5577
BAB1_0626	BR0602	BMEI1339	hypothetical protein	3.6877	N
BAB1_0743	BR0725	BMEI1227	hypothetical protein [possible relationship to acetyltransferases]	0.6340	0.6160
BAB1_0992	BR0973	BMEI1005	hypothetical protein	1.1191	0.4853
BAB1_1106	BR1083	BMEI0899	COG3617; prophage antirepressor [possible function in transcription]	1.2815	0.6963
BAB1_1194	BR1173	BMEI0813	hypothetical protein	1.2322	0.3799
BAB1_1204	BR1181	BMEI0806	hypothetical protein	1.3258	0.4248
BAB1_1262	BR1241	BMEI0751	hypothetical protein	N	1.5608
BAB1_1390	BR1370	BMEI0633	COG0239; possible integral membrane protein	N	1.8630
BAB1_1392	BR1372	BMEI0631	COG3467; predicted flavin nucleotide binding protein [general function prediction only]	N	1.4038
BAB1_1743	BR1730	BMEI0308	hypothetical protein	0.6744	N
BAB1_1748	BR1736	BMEI0304	COG1607, Acyl-CoA hydrolase [Lipid metabolism]	N	1.3140
BAB1_1939	BR1938	BMEI0127	hypothetical protein	0.4634	0.4569
BAB1_2041	BR2041	BMEI0030	DUF188 superfamily	2.6421	0.5280
BAB2_0160	BRA0162	BMEII1078	COG4893; uncharacterized protein conserved in bacteria	0.6152	1.1670
BAB2_0170	BRA0175	BMEII1067	DUF465 superfamily; putative cytosolic protein	0.2584	N
BAB2_0761	BRA0476	BMEII0789	hypothetical protein	0.5177	N
BAB2_0726	BRA0512	BMEII0758	Pfam01873; YbgT-like protein	0.0079	0.0001
BAB2_0470	BRA0769	BMEII0522	Pfam05635; family of hypothetical proteins	0.0002	0.0025
BAB2_0450	BRA0787	BMEII0503	hypothetical protein	0.0435	0.0180
BAB2_0415	BRA0819	BMEII0468	hypothetical protein	1.0595	1.2254
BAB2_0400	BRA0836	BMEII0454	hypothetical protein	1.0501	1.8400
BAB2_0232	BRA1000	BMEII0296	hypothetical protein	N	0.9205
BAB2_1072	BRA1113	BMEII0186	pemK family protein [possible regulator]	5.1792	0.5605
BAB2_1087	BRA1130	BMEII0169	Hypothetical protein [flagellar locus]	N	1.0841
BAB2_1136	BRA1177	BMEII0118	DUF1634 superfamily	0.2300	0.4828

[a] *ORF number from NCBI.*

[b] *Followed B. suis 1330 assignment.*

[c] *Competitive index (CI): Calculated as Mean Mutants/WT from at least three experiments. N: Not tested.*

[d] *Competitive index (CI): Calculated as Mean Mutants/WT in spleen of 2–5 mice. N: Not tested.*

[e] *A previously characterized virB1 mutant (BA41) serves as attenuation phenotype control.*

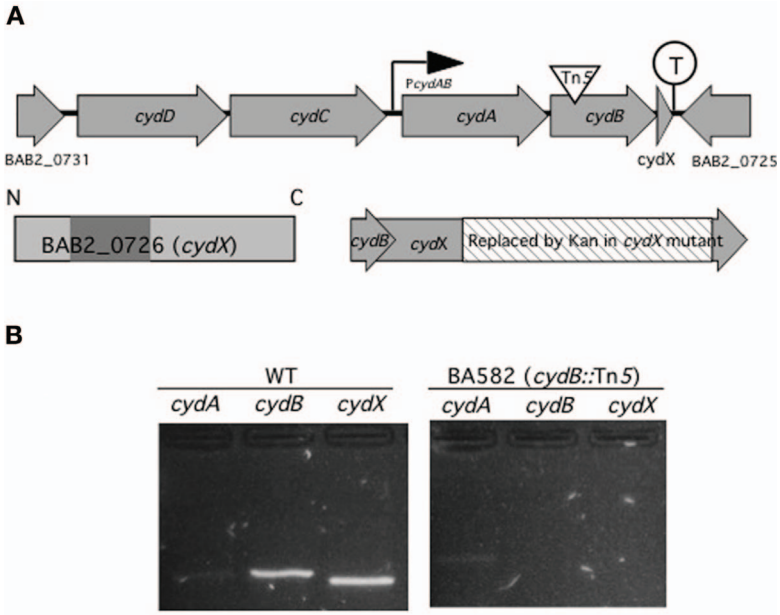

FIGURE 1 | *cydA, cydB, and cydX* are organized and transcribed as a gene cluster. (A) Genetic map of the *cyd* locus in *B. abortus* 2308 chromosome II. *cydA* and *cydB* encode two proteins with similarity to subunit I and II of cytochrome *bd*-1 terminal oxidase in *E. coli*. Upstream of *cydA* and *cydB* are two predicted ABC transporter genes with similarity to *E. coli cydD* and *cydC*. Overlapping 23 bp with *cydB*, *cydX* encodes a 64 aa protein with one predicted transmembrane domain (shaded), spanning residues 17–35. While no promoter is predicted in front of *cydD* there is a promoter (P_{cydAB}), possibly driving transcription of *cydA*, *cydB*, and *cyd*X. A transcriptional terminator (circled T) is located between *cydX* and its adjacent open reading frame BAB2_0725. In BA582 (*cydB*::Tn*5*) a transposon is inserted in *cydB* (triangle) while in *cydX*::Kan part of *cydX* (hatched) is replaced by a kanamycin resistance determinant without disrupting *cydB*. **(B)** Analysis of the *cyd* gene cluster by RT-PCR. PCR products for the indicated primers with wild type *(WT) or cydB*::Tn5 *mutant* (BA582) as a template are shown. The sizes of the predicted amplicons for *cydA*, *cydB*, and *cydX* are 247 bp, 243 bp, and 204 bp, respectively. Control reactions in which reverse transcriptase were omitted had no amplification product (not shown).

To determine whether CydX is functionally related to cytochrome *bd* oxidase, we determined whether deletion of *cydX* affects stationary phase viability, a phenotype that is defective in a *B. abortus cydB* mutant (Endley et al., 2001). As shown in **Figure 2A**, when grown in minimum medium with starting $OD_{600} = 0.05$ all *Brucella* strains replicated equally until they reached stationary phase (48–72 h). However, similar to the *cydB* mutant BA582, the viability of the mutant deleted for *cydX* declined rapidly as the incubation time progressed, while wild type *B. abortus* survived for up to five days. Similar results were observed when the mutants were grown in rich medium (TSB) (data not shown). Our finding that deletion of *cydX* impaired intracellular growth (**Table 2**) and led to loss of viability in stationary phase (**Figure 2A**) prompted us to determine whether this mutant shared other defects associated with inactivation of cytochrome *bd* oxidase (Endley et al., 2001). Similar to a *cydB* mutant, the *B. abortus* mutant lacking *cydX* exhibited significantly ($P < 0.01$) increased sensitivity to hydrogen peroxide compared with wild type *B. abortus* (**Figures 2C,D**).

Disruption of cytochrome *bd* oxidase increases sensitivity to the respiratory chain inhibitor sodium azide and the uncoupling agent nickel in both *Brucella* and *E. coli* (Wall et al., 1992; Edwards et al., 2000; Endley et al., 2001). Similarly to the *B. abortus cydB* mutant, the mutant lacking *cydX* was highly sensitive to the combination of sodium azide plus nickel sulfate, compared to wild type *B. abortus* (**Figure 2E**). The data suggested that the small protein CydX is required for the function of cytochrome *bd* oxidase in *B. abortus*.

COMPLEMENTATION OF THE *CYDX* MUTANT LEADS TO PARTIAL RESTORATION OF *CYD* PHENOTYPES AND IMPROVES INTRACELLULAR GROWTH IN MOUSE MACROPHAGES.

To confirm that the phenotypes of the *cydX*::Kan mutant result from deletion of *cydX*, we conducted complementation experiments to satisfy the molecular Koch's postulates (Falkow, 1988). To this end, constructs encoding either CydX or CydX::3×-FLAG were generated. The *groEL* promoter, known to be induced during growth in macrophages (Lin and Ficht, 1995), was used to drive *cydX* expression. As a control for the expression of *cydX*, we expressed the gene encoding the fluorescent protein DsRed from the same promoter and confirmed fluorescence of the recombinant strain (not shown). Immunoblot analysis of *Brucella* expressing CydX::FLAG with anti-Flag antibody revealed a major band of 10 kDa, a size matching the predicted size of CydX (8 kDa) and 3×FLAG (2 kDa), indicating that CydX is indeed expressed as protein in *Brucella* (not shown). Both the *cydX* mutant and BA582 (*cydB*::Tn*5*) were transformed with constructs expressing *cydX*, *cydB*, or *cydBX* to determine whether the phenotypes associated with cytochrome *bd* oxidase deficiency could be complemented (**Figure 3**). Complementation of both *cydB* and *cydX* mutants with their respective genes restored stationary phase viability (**Figure 3A**), and complementation of

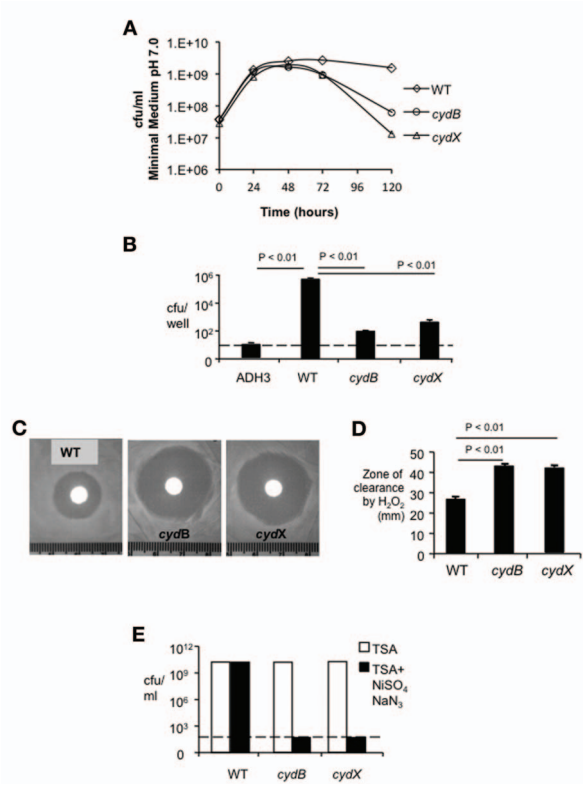

FIGURE 2 | Characterization of the *cydX* mutant. (A) Loss of viability in stationary phase. Growth kinetics of *B. abortus* in modified E medium pH 7.0 was monitored by CFU counting at each indicated time point. Each data point represents results of a single assay that was repeated twice. **(B)** Intracellular survival of the *cydX* mutant in J774 macrophages. Strain ADH3 (*virB2*) served as an attenuated control. Data are represented as the mean ± standard deviation (SD) of triplicate samples from one assay that was repeated twice with the same result. Significance of differences was analyzed by One-Way analysis of variance (ANOVA), with a *P* value of less than 0.05 considered as significant. The dotted line indicates the limit of detection. **(C** and **D)** Sensitivity to hydrogen peroxide. Log-phase bacteria were plated on TSA and oxidative killing was determined by measuring the diameter of clear zone surrounding a paper disk soaked with 5 µl of 30% hydrogen peroxide. Large zones of bacterial growth inhibition were observed in both *cydX* and *cydB* mutants (Typical photographs were shown). Data in **(D)** are represented as means of a single assay with five samples ± SD, that was repeated once with the same result. Significance of differences was analyzed by One-Way ANOVA, with *P* values for pairwise comparisons with $P < 0.05$ indicated. **(E)** Sensitivity to sodium azide plus nickel sulfate. Log-phase bacteria growing TSA or TSA supplemented with 0.15 mM $NiSO_4$ and 0.15 mM NaN_3 were serially diluted and were plated on TSA for enumeration of surviving bacteria. Survival of treatment with sodium azide plus nickel sulfate was calculated as the ratio of CFU on TSA + $NiSO_4$ /NaN_3 to CFU on TSA. While these ratios were approximately 1 for wild type *B. abortus*, neither *cydX* mutant nor the *cydB* mutant survived in the presence of these chemicals. A dotted line indicates the limit of detection of this assay.

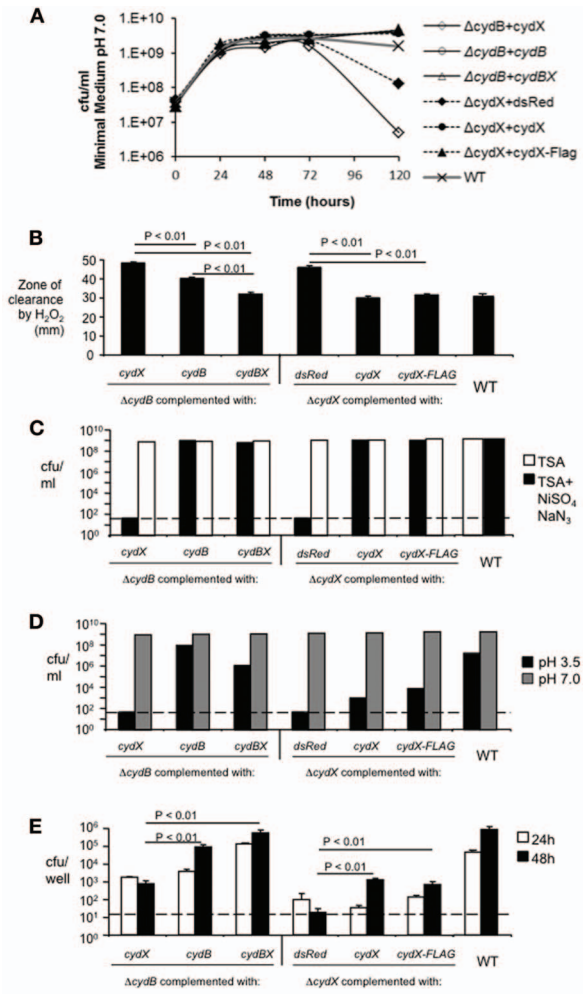

FIGURE 3 | Complementation of *cydX* mutation. Plasmid-encoded copies of *cydB* or *cydBX*, *cydX or cydX-FLAG* were introduced into *cydX* and *cydB* mutants. A plasmid expressing DsRed served as a negative control. **(A)** Restoration of stationary phase viability was determined as decribed in **Figure 2**. **(B)** Sensitivity to 3% Hydrogen peroxide was assayed as described in **Figure 2**. Significance of differences was analyzed by One-Way ANOVA, with *P* values for pairwise comparisons with $P < 0.05$ indicated. **(C)** Sensitivity to sodium azide plus nickel sulfate was assayed as described in **Figure 2**. **(D)** Sensitivity to acid pH. Viability of cultures incubated for 5 h in E-medium adjusted to pH 7.0 or pH 3.5 was assayed as described in the Materials and Methods. Each data point represents results of a single assay that was repeated twice. **(E)** Survival in J774 mouse macrophages. J774 cells were infected with *cydX* or *cydB* mutants containing indicated plasmids. Cells were lysed at 24 and 48 h post-infection. Bacterial CFU were determined by serial dilution and plating on TSA. Data represent the mean of triplicate samples ±SD from a single experiment of three replicates with similar results. Significance of differences was analyzed by One-Way ANOVA, with *P* values for pairwise comparisons with $P < 0.05$ indicated. Dotted lines indicate the limit of detection.

this phenotype in the *cydX* mutant was unaffected by addition of the 3X-FLAG tag to the C terminus of CydX. As expected, the construct expressing DsRed failed to complement the *cydX* mutant since it expressed an irrelevant control protein.

Complementation of hydrogen peroxide sensitivity in the *cydX* mutant was also observed with constructs expressing CydX or CydX::Flag, as evidenced by the reduced zone of clearance around filter disks containing hydrogen peroxide (**Figure 3B**). Introduction of a construct encoding *cydBX* into the *cydB* mutant (BA582) resulted in greater resistance to H_2O_2 compared to introduction of a construct encoding *cydB* only (**Figure 3B**). This

result suggested that the Tn*5* insertion in *cydB* in BA582 could have a polar effect on expression of *cydX*.

A second phenotype of the *cydX* mutant, lack of growth in the presence of nickel and azide, was also complemented to the wild type level by introduction of an intact copy of *cydX* or *cydX::3x-Flag*, similar to what was observed for complementation of the *cydB* mutant (**Figure 3C**).

Two *cydX* mutant phenotypes, sensitivity to acidic conditions and intracellular survival in J774 macrophages, were only partially complemented by introduction of constructs expressing CydX or CydX::FLAG (**Figures 3D,E**). In the experiment shown in **Figure 3E**, there were no remarkable differences between CydX mutant transformed with pGroEL/dsRed, pGroEL/CydX, or pCydX/Flag at 24 h post-infection. However, at 48 h post-infection, the CFU recovered for mutants complemented with CydX or CydX::Flag were markedly higher than that of mutant transformed with pGroEL/dsRed (control). Complementation of the *cydX* mutant with constructs expressing CydX or CydX::FLAG resulted in partial restoration of intracellular survival, with a net increase in bacterial numbers between 24 and 48 h. In contrast, the *cydX* mutant complemented with the DsRed-expressing construct failed to replicate during this time and actually decreased in numbers. Based on this observation we concluded that the intracellular growth defect of *cydX* mutant with impaired cytochrome *bd* oxidase activity could be partially rescued by providing wild type CydX or CydX::FLAG *in trans*. Interestingly, expression of *cydX* together with *cydB* increased survival of the *cydB* mutant more then complementation with *cydB* alone (**Figure 3E**). However, expression of *cydX* alone in the same copy number did not completely restore intracellular replication of the *cydX* mutant.

It was of interest that while some phenotypes of the *cydX* mutant, such as sensitivity to H_2O_2 or nickel sulfate/sodium azide, could be complemented to the level of the wild type, survival of pH 3.5 or intracellular replication of the *cydX* mutant were restored to a lesser degree. It is possible that these two former phenotypes require only some degree of cytochrome *bd* oxidase activity, whereas intracellular growth and acid resistance represent stress conditions that may require either precise regulation of *cydABX* expression or specific ratios of CydX to CydA and CydB. Since CydX is predicted to be an integral cytoplasmic protein (see below), it is conceivable that free CydX that is not associated with a cytochrome *bd* oxidase complex might insert into the cytoplasmic membrane and destabilize other cell envelope functions that are essential for resistance to acidic pH and intracellular replication. Our finding that expression of *cydX* in a *cydB* mutant lacking cytochrome *bd* oxidase actually decreased stationary survival (**Figure 3A**) is consistent with this interpretation.

THE C TERMINUS OF CydX IS LOCATED IN THE PERIPLASM

CydX is predicted to have a single transmembrane domain of 15–17 residues. Since components of cytochrome *bd* oxidase in *E. coli* are located at the cytoplasmic membrane (Miller and Gennis, 1983; Green et al., 1984; Miller et al., 1988) we hypothesized that CydX could mediate its function by colocalizing with CydAB at the cytoplasmic membrane, where it might serve to tether the enzymatic components of the oxidase. To test this hypothesis, we performed membrane fractionation of *B. abortus*, which failed to yield a cytoplasmic membrane fraction of sufficient purity to give conclusive results (data not shown). As a second approach we constructed N- and C-terminal fusions of CydX to TEM-1 β-lactamase (Zhang et al., 2004) to determine the topology of CydX in the cytoplasmic membrane. To this end, we designed two constructs by cloning *cydX* in front or behind TEM-1 β-lactamase (Datta and Kontomichalou, 1965) with a 3×FLAG epitope replacing the TEM-1 signal sequence. Both constructs of pCydX/FT (FT stands for 3×FLAG::TEM1) and pFT/CydX were DNA sequenced to ensure in-frame expression of Cyd::FT or FT::CydX fusions.

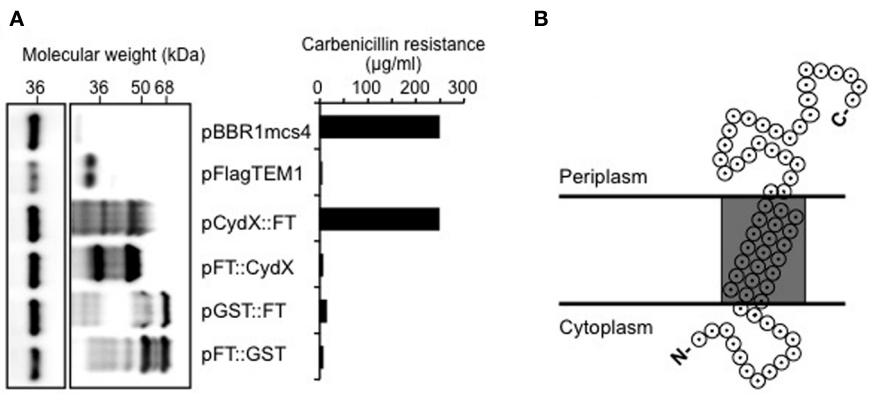

FIGURE 4 | The C-terminus of CydX is located in the periplasm of *B. abortus*. (A) Western Blot showing expression levels of β-lactamase fusions to CydX and Glutathione-S-transferase (GST; a cytoplasmic control). Blots were probed with a monoclonal antibody against TEM-1 β-lactamase (right blot). As a loading control, the *B. abortus* protein Bcsp31 detected using a polyclonal antibody (shown on the left) [Sun et al. (2005)]. **(B)** Maximum concentration of carbenicillin allowing growth of *B. abortus* expressing each β-lactamase fusion protein. Plasmid pBBR1mcs4 is a positive control that expresses β-lactamase resistance. pFlagTEM1 is the plasmid carrying TEM-1 β-lactamase lacking its N-terminal sequence and was used for generating N- and C-terminal fusions to CydX and GST. **(C)** Proposed topological model for CydX in *Brucella* based on current understanding of cytochrome *bd* quinol oxidase in *E. coli* and predicted single transmembrane domain (shaded area) in CydX protein sequence.

Since β-lactamase only confers resistance to β-lactam antibiotics if it localizes to the periplasm, we could conclude based on ampicillin resistance of each recombinant strain whether the TEM-1 domain was localized in the periplasm. As a control for a resident cytosolic protein we constructed a Glutathione S-transferase (GST) β-lactamase fusions. The GST constructs and pFlagTEM1, which is the empty vector to make CydX β-lactamase fusions, were used as negative controls. As a positive control we used pBBR1MCS4, which encodes β-lactam resistance. Expression of CydX::FT allowed *Brucella* to survive a concentration of up to 250 μg/ml, of carbenicillin, a maximal concentration similar to that of positive control pBBR1MCS4, whereas the other strains tested were sensitive to 20 μg/ml of carbenicillin (**Figure 4A**). This result indicated that the β-lactamase moiety of CydX::FT was located in the periplasm, while in FT::CydX, TEM-1 was retained in the cytoplasm. To exclude the possibility that the carbenicillin sensitivity was due to low or absent expression of β-lactamase fusions in those strains, we performed immunoblot analysis of bacterial lysates using a monoclonal antibody specific to TEM-1 β-lactamase (QED Bioscience Inc.). High levels of β-lactamase fusion protein expression were detected in all but the pBBR1MCS4 transformed *Brucella*, which could be attributed to the lower expression from a normal promoter, compared to the IPTG inducible trc promoter in the other constructs, since the TEM-1 constructs share identical sequences to the *bla* gene in pBBR1MCS4 (**Figure 4A**). Based on our current understanding about cytochrome *bd* oxidase in other organisms we predict that *Brucella* CydX is inserted in cytoplasmic membrane, with its N terminus at the cytosolic face and C terminus in the periplasm, as depicted in **Figure 4B**.

In conclusion, we have identified a small protein, CydX (BAB2_0726) that is co-regulated with CydAB, and is required for full function of cytochrome *bd* oxidase in *B. abortus*. This terminal oxidase is used by *Brucella* spp. to survive inside eukaryotic cells, where it contributes to intracellular replication (Endley et al., 2001; Loisel-Meyer et al., 2005; Wu et al., 2006). Interestingly, in contrast to *B. abortus*, in which *cydB* mutants were highly attenuated, *B. suis cydB* mutants were hypervirulent, suggesting different use of terminal oxidases in these two species (Jimenez de Bagues et al., 2007). Additional evidence for CydX expression during *in vitro* growth of *B. abortus* was recently provided by a proteomic study (Lamontagne et al., 2010). The requirement of CydX for cytochrome *bd* oxidase function may be shared with other bacteria expressing cytochrome *bd* oxidase, such as *E. coli* and *S. Typhimurium*, since a small protein YbgT was identified downstream of the *cydB* gene in these two organisms (Alix and Blanc-Potard, 2009; Hemm et al., 2010). In *E. coli*, cytochrome *bd*-I oxidase was shown to assemble into large respiratory domains in the cytoplasmic membrane (Lenn et al., 2008), so the insertion of CydX into the cytoplasmic membrane suggests that it might play a role in organizing these complexes at the cytosolic face of the cytoplasmic membrane or might be required for their terminal oxidase activity. Further experimentation will be needed to determine the precise mechanism by which CydX contributes to cytochrome *bd* oxidase function.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX

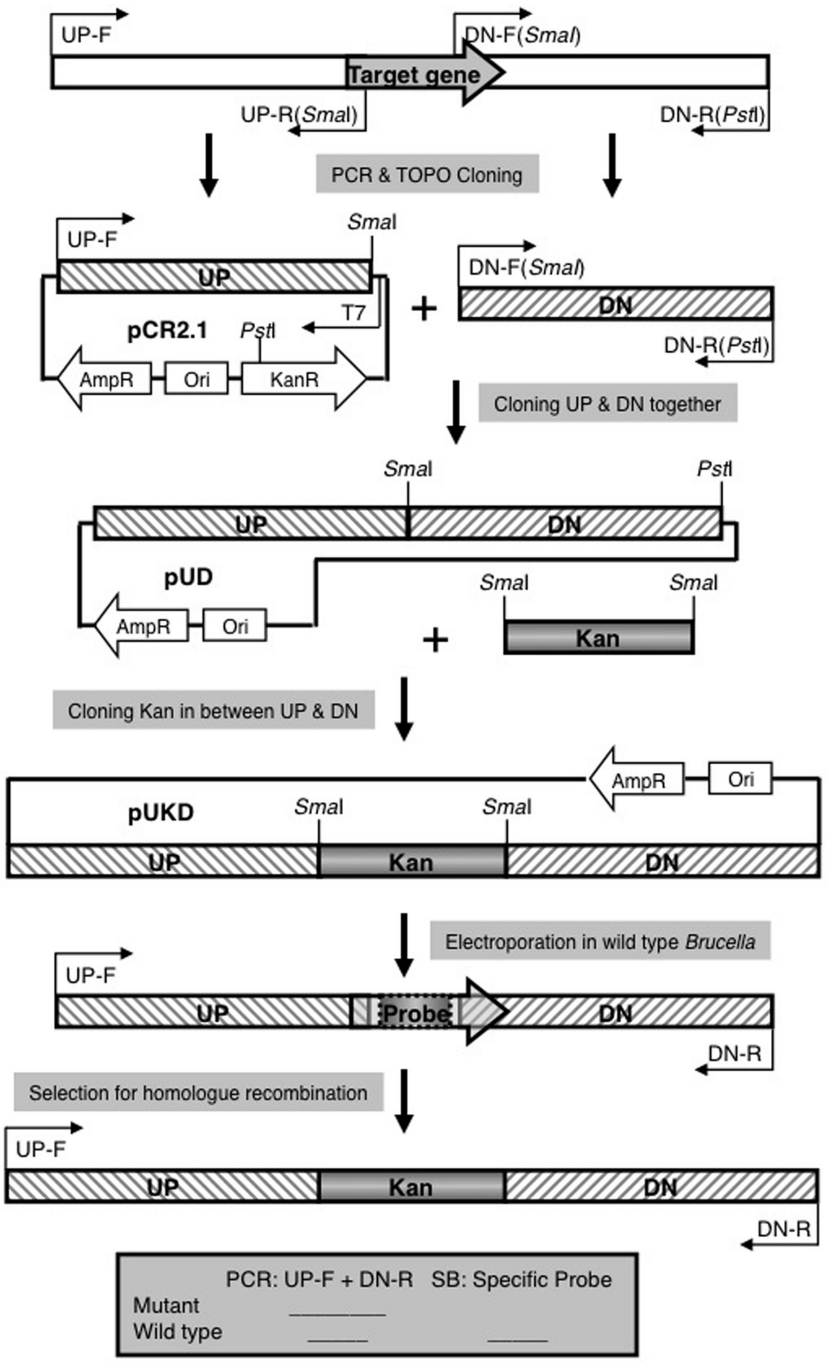

FIGURE A1 | Strategy for Allelic Exchange Mutagenesis. See Materials and Methods for explanation.

Table A1 | Primers used in this work.

Primer	Sequence*	Description
GBRORF0459 DN-F	TCCCCCGGGAAAATAATTGACCATGGCΔ	ΔBAB1_0061
GBRORF0459 DN-R	AACTGCAGGGGCAGGCCCGCCGCCT	
GBRORF0459 UP-F	GTGCACCGCATTTATTGCGT	
GBRORF0459 UP-R	TCCCCCGGGTTGCAGATCAAAGAGCG	
GBRORF0206 DN-F	TCCCCCGGGAGCCTGCGTTATGCTGCCCGG	ΔBAB1_0355
GBRORF0206 DN-R	AAACTGCAGCACCACGAAAATGGCCGTG	
GBRORF0206 UP-F	GCAGGCGCTCAAAATAATGTG	
GBRORF0206 UP-R	TCCCCCGGGCTTTGCGGGGGGCGTTCA	
GBRORF0049 DN-F	TCCCCCGGGATAGGTGTGCGCCCCGC	ΔBAB1_0513
GBRORF0049 DN-R	AACTGCAGAAGTCGAATCCGCCCTC	
GBRORF0049 UP-F	CCGGCCTTCATGAGCCGGAT	
GBRORF0049 UP-R	TCCCCCGGGTCGTTTTGCGGGCCAAC	
GBRORF2128 DN-F	TCCCCCGGGCCGTTGTTATAAACGACGAC	ΔBAB1_0581
GBRORF2128 DN-R	AAACTGCAGAGCTTTTACCAAAGAACAGG	
GBRORF2128 UP-F	GGCTCATACCCGCCAAATGCA	
GBRORF2128 UP-R	TCCCCCGGGTTCAGCCGTTCCAGCACTT	
GBRORF2083 DN-F	TCCCCCGGGAGAAGAAACTGTTGTT	ΔBAB1_0626
GBRORF2083 DN-R	AAACTGCAGGTCCTTGTCGTCTTCAACA	
GBRORF2083 UP-F	CTGCTCAGATCGCCGGATGGC	
GBRORF2083 UP-R	TCCCCCGGGATTTATTGGCTGTCATATTGTT	
GBRORF1960 DN-F	TCCCCCGGGACGACCTGCTTTCGCAGAT	ΔBAB1_0743
GBRORF1960 DN-R	AAACTGCAGCCCGTATGTAACAATGAG	
GBRORF1960 UP-F	CCTATGCAGCTTCCACCATTG	
GBRORF1960 UP-R	TCCCCCGGGTAAGTTTGTGTGCTCATTC	
GBRORF1747 DN-F	TCCCCCGGGCTATCCCGCGACGAGCG	ΔBAB1_0960
GBRORF1747 DN-R	AAACTGCAGAAGCTGATCTTCATCGAT	
GBRORF1747 UP-F	GATAGTGCCAGGATAAGGAAA	
GBRORF1747 UP-R	TCCCCCGGGATGGAAAACTTCATCATT	
GBRORF1716 DN-F	TCCCCCGGGTCAAGAAATTCAACCTT	ΔBAB1_0992
GBRORF1716 DN-R	AACTGCAGATCGCCTGATGGCAATC	
GBRORF1716 UP-F	TCCGATTGGGCGTAGCTTAC	
GBRORF1716 UP-R	TCCCCCGGGTACTGGCGCTGGCGTCT	
GBRORF1608 DN-F	TCCCCCGGGACGCTCATCACCTTGACGGA	ΔBAB1_1106
GBRORF1608 DN-R	AAACTGCAGCCCTCCCGCCCCTCACGT	
GBRORF1608 UP-F	GGCGAGAGGGATGAAGATCGA	
GBRORF1608 UP-R	TCCCCCGGGTCCATGAAGTTGAAGATTT	
GBRORF1520 DN-F	TCCCCCGGGCGATTCCATTAAAACAG	ΔBAB1_1194
GBRORF1520 DN-R	AACTGCAGAATCATGGCTCTAAATC	
GBRORF1520 UP-F	CCTTGGCATGGCGCTGCGTC	
GBRORF1520 UP-R	TCCCCCGGGCCTGTCCTTCGCTCGGG	
GBRORF1512 DN-F	TCCCCCGGGCGTTGCCGTCGGCCATAG	ΔBAB1_1204
GBRORF1512 DN-R	AACTGCAGCCTGCGGTCCATTCGTA	
GBRORF1512 UP-F	GTTGTGCTACTTCATTTACG	
GBRORF1512 UP-R	TCCCCCGGGTCGCCGCACCGCCTG	
GBRORF1452 DN-F	TCCCCCGGGCGACGACGAAGAGGAAGAG	ΔBAB1_1262
GBRORF1452 DN-R	AAACTGCAGACGCCTTCGTTCATCT	
GBRORF1452 UP-F	CAGTGCAGGAACCTTATTGCG	
GBRORF1452 UP-R	TCCCCCGGGACTCGGATGTGCTTCAATAGC	
GBRORF1325 DN-F	TCCCCCGGGTTTGCCGCATGGCTCGACGC	ΔBAB1_1390
GBRORF1325 DN-R	AAACTGCAGCGACGGAGTGGCGGCTCT	

(Continued)

Table A1 | Continued

Primer	Sequence*	Description
GBRORF1325 UP-F	GGCCAGATAGCCCGGAACGA	
GBRORF1325 UP-R	TCCCCCGGGACCCACATAACATCTAAAGGA	
GBRORF1323 DN-F	TCCCCCGGGCATCAGGCGGAGTGACC	ΔBAB1_1392
GBRORF1323 DN-R	AAACTGCAGGACGTCGGCCTCCATTCTC	
GBRORF1323 UP-F	GCTTGTGACGCTGGAGGAAAA	
GBRORF1323 UP-R	TCCCCCGGGTCCGACATTTCCGTTATCAAC	
GBRORF0969 DN-F	ACCCCCGGGCTTTCACCTGCACGAAG	ΔBAB1_1743
GBRORF0969 DN-R	ACCTGCAGACCTATGGCGTGGATTCG	
GBRORF0969 UP-F	ACGCGCGATGGCTCAATGC	
GBRORF0969 UP-R	ACCCCCGGGCAGGATTTTGGTCAC	
GBRORF0964 DN-F	TCCCCCGGGTATCCCCATCGAGCTTGCC	ΔBAB1_1748
GBRORF0964 DN-R	AAACTGCAGGATCGCCCGAATGCGATT	
GBRORF0964 UP-F	CCGACACCCAGATTCATGCGC	
GBRORF0964 UP-R	TCCCCCGGGTTCTTGAAAACGTCTTTCATG	
GBRORF0765 DN-F	TCCCCCGGGTTGATGTCCTTCAATTC	ΔBAB1_1939
GBRORF0765 DN-R	AACTGCAGGATGCGCCAGACGAA	
GBRORF0765 UP-F	ACGGCGCATGCTTCTTGTCG	
GBRORF0765 UP-R	TCCCCCGGGGGGCTGGTTCGGACAAT	
GBRORF0663 DN-F	TCCCCCGGGCAATACGTTCCGGCGAAG	ΔBAB1_2041
GBRORF0663 DN-R	AACTGCAGGTGTCGAGAAGCTCAAG	
GBRORF0663 UP-F	CCTGCATCTGCAAAGGCC	
GBRORF0663 UP-R	TCCCCCGGGTGGTTGATGCCATCATT	
GBRORFA0247 DN-F	TCCCCCGGGTGTGCGCGGCCAAGATAA	ΔBAB2_0160
GBRORFA0247 DN-R	AAACTGCAGCCCTGCAAAATTTCCTGT	
GBRORFA0247 UP-F	CCATGGGTATGATGATGCTGC	
GBRORFA0247 UP-R	TCCCCCGGGTTTCCCGCAAGCTGCAAGC	
GBRORFA0258 DN-F	TCCCCCGGGCGCTTGCCGCCGCCTGATT	ΔBAB2_0170
GBRORFA0258 DN-R	AAACTGCAGATTGGTGAGGGGGCGAAA	
GBRORFA0258 UP-F	CCGCTTCGTCGAACAGGCAAC	
GBRORFA0258 UP-R	TCCCCCGGGGCGTATTCGACATTCTAC	
GBRORFA1086 DN-F	TCCCCCGGGCTTTCCATCGCCTGAAACTGT	ΔBAB2_0232
GBRORFA1086 DN-R	AAACTGCAGGGGAAATGTAGGAGGTAG	
GBRORFA1086 UP-F	GAAGAAGGCCTTATCTGATCA	
GBRORFA1086 UP-R	TCCCCCGGGCCGCCCCCTCTTCACCATGA	
GBRORFA0924 DN-F	TCCCCCGGGTGGAACACTCTCCCTGC	ΔBAB2_0400
GBRORFA0924 DN-R	AACTGCAGTCCTTTGCTGCCTTTGT	
GBRORFA0924 UP-F	GCGTATCAGCCATGCAGGCG	
GBRORFA0924 UP-R	TCCCCCGGGTAAGACGTAGGTTAGGC	
GBRORFA0907 DN-F	TCCCCCGGGCAGCAATGGTTCGACGAAAG	ΔBAB2_0415
GBRORFA0907 DN-R	AAACTGCAGACAAATGCACCGAGGAT	
GBRORFA0907 UP-F	GCGGATCGGCGAGACTTTTCC	
GBRORFA0907 UP-R	TCCCCCGGGCAGTTCCATGACCCGTTCT	
GBRORFA0873 DN-F	TCCCCCGGGCGCTTCCCGCCTACTACTGA	ΔBAB2_0450
GBRORFA0873 DN-R	AAACTGCAGCCATAATGTCCGCAAAC	
GBRORFA0873 UP-F	GGTTTTGCAGTGGCTTCCAG	
GBRORFA0873 UP-R	TCCCCCGGGGGCAGCAAACTGGACATT	
GBRORFA0854 DN-F	TCCCCCGGGCTTCTCATCCGCAAGCTA	ΔBAB2_0470
GBRORFA0854 DN-R	AAACTGCAGATAGCGCGGCTTGTGCAC	
GBRORFA0854 UP-F	CATGTTCAAGCGCACGATCCG	
GBRORFA0854 UP-R	TCCCCCGGGATATGAATTGATTGACATCAC	

(Continued)

Table A1 | Continued

Primer	Sequence*	Description
GBRORFA0598 DN-F	TCCCCCGGGCAACAAGCACCACTGAAATG	ΔBAB2_0726
GBRORFA0598 DN-R	AAACTGCAGATATGGCCCGGCGCGGCAT	
GBRORFA0598 UP-F	CGGCGGCTTCGTGCCGGCGC	
GBRORFA0598 UP-R	TCCCCCGGGTTGCTCTCATCCTCGATCA	
GBRORFA0563 DN-F	TCCCCCGGGCCTATGCAGAAGCCCTG	ΔBAB2_0761
GBRORFA0563 DN-R	AACTGCAGAAGAGAATATGGCCGTC	
GBRORFA0563 UP-F	CGCTTTTTCCCGGCAGCATG	
GBRORFA0563 UP-R	TCCCCCGGGTTCGGTTTCAGTTTCTC	
GBRORFA0429 DN-F	TCCCCCGGGCTGAAGCAGAACTCAAGAAAGTT	ΔBAB2_0862
GBRORFA0429 DN-R	AAACTGCAGTTTAATGAGATATTTC	
GBRORFA0429 UP-F	CAATGCAGGCGCAATTGCAAC	
GBRORFA0429 UP-R	TCCCCCGGGAAGAGAGCCTTAATCATTTCC	
GBRORFA1199 DN-F	TCCCCCGGGTTCCCGTTTCTGCGACG	ΔBAB2_1072
GBRORFA1199 DN-R	AAACTGCAGATAACGGCATCAGCACCG	
GBRORFA1199 UP-F	GGGCACTTCTTCTGCCACGT	
GBRORFA1199 UP-R	TCCCCCGGGATAGTTTCGGCTTCGAT	
GBRORFA0014 DN-F	TCCCCCGGGATGAGTCGCAAAAGCGGAG	ΔBAB2_1087
GBRORFA0014 DN-R	AAACTGCAGGGTTGCCGGCGCCTCTG	
GBRORFA0014 UP-F	GGCCGCCGTGCTGGCGTTTTG	
GBRORFA0014 UP-R	TCCCCCGGGCGTGTTCCAGCCTGCGTCAT	
GBRORFA0062 DN-F	TCCCCCGGGTAGGCTTATCCAGGCTGTA	ΔBAB2_1136
GBRORFA0062 DN-R	AAACTGCAGGCCTATGGTCTCTTCCCGG	
GBRORFA0062 UP-F	CCGCTTCTCACATCCATTTTC	
GBRORFA0062 UP-R	TCCCCCGGGCGATGCTGTTATCTTCGAT	
GroEL-P-F	AGCATGCCGTTCGGGCGATGGAAACAGGCGTG	Promoter P_{GroEL}
GroEL-P-R	ACATATGGTATAACCCTTGGTGTTATAGACG	
dTomato-F	ACATATGGTGAGCAAGGGCGAGGAGGTC	pGroEL/dsRed
dTomato-R	ACCCGGGATGCATTACTTGTACAGCTCGTCCATGCCGTAC	
A0598-Flag-F	ACATATGAGAGCAACCACGCTTAC	pGroEL/CydX
A0598 -PstI	ACTGCAGTCAGTGGTGCTTGTTGCCTTC	
CydB-F-NdeI	ACATATGATACTCAGCGATTTGTTGGACTATC	pGroEL/CydB
CydB-R-PstI	ACTGCAGTCAGTAAGCGTGGTTGCTCTCATCCTC	
CydB-F-NdeI	ACATATGATACTCAGCGATTTGTTGGACTATC	pGroEL/CydBX
A0598 -PstI	ACTGCAGTCAGTGGTGCTTGTTGCCTTC	
A0598-Flag-F	ACATATGAGAGCAACCACGCTTAC	pCydX/FT
A0598-Flag-R	AGTCGACGTGGTGCTTGTTGCCTTC	
A0598 -XbaI	ATCTAGAAGAGCAACCACGCTTACTGATTG	pFT/CydX
A0598 -PstI	ACTGCAGTCAGTGGTGCTTGTTGCCTTC	
Promoter-F	TTAGGGTACCGTTAACGCTCCCCGAAAACGGC	Promoter $P_{BMEII0192}$
Promoter-R	AGTCGACATATGTGATTTCCGGC	
Flag-F	ACATATGAGCCTAGTCGACTACAAAGACCATGAC	pBBR1Flag
Flag-R	AGAGCTCCTGCAGTTACTATTTATCGTCGTCATC	
A0598-Flag-F	ACATATGAGAGCAACCACGCTTAC	pCydX/Flag
A0598 -PstI	ACTGCAGTCAGTGGTGCTTGTTGCCTTC	

**Engineered restriction sites underlined.*